NEW GDF-9 AND GDF-9B (BMP-15) SEQUENCES FOR ALTERING MAMMALIAN OVARIAN FUNCTION AND OVULATION RATE

TECHNICAL FIELD

The present invention relates to new sequences for altering mammalian ovarian function and ovulation rate.

In particular, the invention broadly concerns a novel mutation in the GDF-9 gene and two novel mutations in the GDF-9B gene. These mutations have been found to be involved in increasing the ovulation rate in heterozygous female mammals; or causing sterility in homozygous female mammals.

BACKGROUND ART

The genes GDF-9 and GDF-9B (also known as BMP15) code for proteins which are expressed exclusively in the oocyte of the developing follicle, and which play an essential role in mammalian fertility. GDF-9 is a member of the transforming growth factor beta (TGFß) superfamily (McPherron and Lee, 1993) which is expressed in oocytes from the primary stage of follicular development until ovulation (McGrath *et al.*, 1995; Laitinen *et al.*, 1998). GDF-9B is closely related to GDF-9 (Dube *et al.*, 1998; Laitinen *et al.*, 1998) and is expressed in mouse oocytes at the same time as GDF-9, but in human primary follicles slightly later than GDF-9. In the ovary GDF-9 and GDF-9B have now been shown to be expressed exclusively in the developing oocyte in humans (Aaltonen *et al.*, 1999), rodents (Laitinen *et al.*, 1998; Dube *et al.*, 1998; Jaatinen *et al.*, 1999), ruminants (Bodensteiner *et al.*, 1999; Bodensteiner *et al.*, 2000; Galloway *et al.*, 2000) and marsupials (Eckery *et al.*, 2002). In sheep expression of GDF-9 can be seen in primordial follicles whereas GDF-9B is expressed in primary follicles (Bodensteiner *et al.*, 1999; Galloway *et al.*, 2000).

GDF-9 is an essential growth factor for folliculogenesis in mice. Female GDF-9 knockout mice (GDF-9 -/-) are infertile due to a block in follicular development at the primary stage (Dong *et al.*, 1996). GDF-9B does not appear to be crucial for mouse folliculogenesis as knockout female mice (BMP15 -/-) are fertile (Yan *et al.*, 2001), even though fecundity is somewhat reduced. However, GDF-9B is essential for folliculogenesis in sheep as those carrying two copies of naturally-occurring inactivating GDF-9B mutations are infertile due to a block in follicular development at the primary stage (Galloway *et al.*, 2000).

In sheep it is also clear that heterozygotes carrying inactivating mutations in one copy of GDF-9B (whereby only one copy of the gene produces active protein) have an increased ovulation rate (Galloway *et al.*, 2000). A similar increase in ovulation rate in heterozygote mice with knockouts in either GDF-9 or GDF-9B has not been observed (Yan *et al.*, 2001). Double knockouts of both GDF-9 and GDF-9B in mice are infertile with a similar phenotype to GDF-9 -/- mice alone, but GDF-9B knockout mice (BMP15 -/-) with one active copy of GDF-9, have a lower fecundity than BMP15 -/- females (Yan *et al.*, 2001), suggesting that the relative dose of these gene products may also play a role in mice. Collectively these findings suggest that important differences exist in the actions of GDF-9 and GDF-9B between species with a high ovulation rate phenotype (e.g. mice, rats) and those with a low ovulation rate phenotype (e.g. sheep, humans).

GDF-9 maps to a region of sheep chromosome 5 (Sadighi et al., 2002) which is syntenic to the map locations for GDF-9 on human chromosome 5 and mouse chromosome 11. GDF-9B maps to the sheep X chromosome (Galloway et al., 2000) in a region of the chromosome syntenic to the map locations for GDF-9B on the human and mouse X chromosomes (Dube *et al.*, 1998; Aaltonen *et al.*, 1999).

GDF-9 and GDF-9B, like other members of the TGFß family, are coded as prepropeptides containing a signal peptide, a proregion and a C-terminal mature region which is the biologically active peptide. Cleavage of the mature region from the proregion is carried out by an intracellular furin-like protease, and occurs at a conserved furin protease cleavage site. Members of the TGFß superfamily are biologically active as dimers, and although GDF-9 and GDF-9B do not contain the cysteine molecule responsible for covalent interchain disulphide bonding seen in other members of the family, these molecules are thought to be biologically active as dimers (Galloway *et al.*, 2000; Yan et al., 2001). However it is unclear whether the physiologically active dimers are homodimers (GDF-9-GDF-9 and GDF-9B-GDF-9B), or heterodimers (GDF-9-GDF-9B) or whether all three dimer forms play a role. It has been postulated based on the above models that GDF-9 homodimers play a more important role in the mouse but in sheep the GDF-9B homodimers are the most bioactive (Yan *et al.*, 2001). It is unclear whether any such difference is related to the fact that sheep are mono-ovulatory animals (maturing usually only one egg per cycle) whereas mice are poly-ovulatory. Clearly both GDF-9 and GDF-9B play crucial roles in controlling and maintaining fertility in mammals, and understanding the nature of their actions is essential for the development of therapies.

GDF-9 and GDF-9B in sheep

The sheep GDF-9 gene spans about 2.5 kb and contains 2 exons separated by a single 1126 bp intron (Bodensteiner *et al.*, 1999). The full length coding sequence is 1359 nucleotides long and encodes a pre-propeptide of 453 amino acid residues (Genbank accession number AF078545). A pre-pro region of 318 residues includes a predicted signal sequence, and ends with the RHRR furin protease cleavage site at residues 315 – 318. Residues 319 to 453 beyond the cleavage site code for the 135 amino acid mature

active peptide. The amino acid sequence of the sheep GDF-9 mature peptide is 92.8 % similar to the human mature peptide and 87.1 % similar to the mouse mature peptide.

Sheep GDF-9B has previously been sequenced by us (Galloway *et al.* 2000; Genbank accession nos. AF236078, AF236079) and has a very similar gene structure to GDF-9. The full length coding sequence of 1179 nucleotides is contained in two exons, separated by an intron of about 5.4 kb, and encodes a pre-propeptide of 393 amino acid residues. A pre-pro region of 268 residues includes a predicted signal sequence, and ends with the RRAR furin protease cleavage site at residues 265 – 268. Residues 269 to 393 beyond the cleavage site code for a 125 amino acid mature active peptide. The amino acid sequence of the sheep GDF-9B mature peptide is 78.3 % similar to the human mature peptide and 78.6 % similar to the mouse mature peptide.

We have previously shown that the effects on prolificacy in Inverdale and Hanna sheep is due to naturally-occurring mutations in GDF-9B (Galloway *et al.*, 2000). Both Inverdale and Hanna sheep have increased ovulation rates in heterozygous carriers of mutated GDF-9B, but female homozygous carriers are infertile with 'streak' ovaries (Davis *et al.*, 2001). Infertility in these sheep is due to primary ovarian failure caused by the inability of the follicle to develop beyond the primary stage. Hanna sheep have a single C to T mutation at nucleotide 871 of the GDF-9B coding sequence (nucleotide 67 of the mature GDF-9B peptide coding region) which produces a premature stop codon in the place of a glutamic acid (Q) at amino acid residue 291 (residue 23 of the mature protein). Inverdale sheep have a distinct T to A mutation at nucleotide 896 (nucleotide 92 of the mature GDF-9B peptide coding region) which substitutes valine (V) for aspartic acid (D) at residue 299 (residue 31 of the mature peptide). This substitution of a hydrophobic valine with a negatively charged aspartate changes the electrostatic surface potentials of an area involved in dimer formation and appears to disrupt dimerisation and hence abolish biological activity (Galloway *et al.*, 2000).

In addition to the Inverdale and Hanna lines of sheep discussed above, the Cambridge and F700 Belclare strains of sheep have also been shown to carry genes affecting prolificacy as evidenced in high ovulation rate (Hanrahan, 1991) and the presence of sterile ewes with 'streak-like' ovaries (Hanrahan, 1991).

The Cambridge breed was established at the Cambridge University farm in 1964 by screening 54 ewes selected for their high prolificacy from nine British sheep breeds. Ewes within the screened flock were subsequently selected on high litter size. Ewes with the highest ovulation rates were selected from this flock in 1984 to provide the foundation animals for the flock now maintained at Teagasc Sheep Research Centre in Ireland (Hanrahan, 1991). A progeny test of 10 Cambridge rams, descended from the flock in Ireland, gave progeny mean ovulation rates ranging from 2.1 - 4.2.

The Belclare breed was established in 1978 at the Belclare Research Centre of Teagasc in Ireland by crossing three populations of prolific sheep assembled by Teagasc in Ireland. These were Fingalway, High Fertility, and Lleyn sheep (Hanrahan, 1991). The Fingalway was an interbred cross (from F1) of the Finnish Landrace and Galway breeds; the Lleyn is a breed native to north west Wales and selected animals were imported into Ireland in 1975 by Teagasc for the purposed of developing the Belclare breed; the High Fertility was developed in Ireland during the 1960s from ewes with exceptional litter size performance collected from farms in Ireland between 1963 and 1965. A subline of the Belclare (called F700 line) was derived from Belclare sheep that had exceptionally high ovulation rates (Hanrahan 1991). Progeny of 10 Belclare rams had mean ovulation rates ranging from 1.9 – 4.2.

All references, including any patents or patent applications cited in this specification are hereby incorporated by reference. No admission is made that any reference constitutes prior art. The discussion of the references states what their authors assert, and the

applicants reserve the right to challenge the accuracy and pertinency of the cited documents. It will be clearly understood that, although a number of prior art publications are referred to herein, this reference does not constitute an admission that any of these documents form part of the common general knowledge in the art, in New Zealand or in any other country.

It is acknowledged that the term 'comprise' may, under varying jurisdictions, be attributed with either an exclusive or an inclusive meaning. For the purpose of this specification, and unless otherwise noted, the term 'comprise' shall have an inclusive meaning - i.e. that it will be taken to mean an inclusion of not only the listed components it directly references, but also other non-specified components or elements. This rationale will also be used when the term 'comprised' or 'comprising' is used in relation to one or more steps in a method or process.

It is desirable to provide new sequence mutations in the GDF-9B and GDF-9 genes linked to altered fertility rates.

It is an object of the present invention to go some way towards fulfilling this desideratum and/or provide the public with a useful choice.

Further aspects and advantages of the present invention will become apparent from the ensuing description which is given by way of example only.

SUMMARY OF INVENTION

The present invention is concerned with novel mutated GDF-9 and GDF-9B gene sequences which alter mammalian ovarian function and ovulation rate. The invention broadly has application in increasing or decreasing the ovulation rate, or causing sterility in a female mammal, and additionally encompasses regulation of the function of the corpeus luteum.

In particular, the present invention concerns a novel mutation in GDF-9 which increases ovulation rate in heterozygotes and causes sterility in homozygotes for this gene.

The present invention also concerns two mutations in GDF-9B. Heterozygotes for either one of these mutations have an increased ovulation rate. Mammals which are heterozygotes for both mutations in GDF-9B (where each mutation is on a separate X chromosome) are sterile.

Surprisingly, the inventors have also discerned that in female mammals that are heterozygous for the mutated GDF-9 gene and heterozygous for one of the GDF-9B gene mutations, an even higher ovulation rate exists than in mammals heterozygous for one mutation in either GDF-9 or GDF-9B alone.

Knowledge of the mutated gene sequences can be applied to a test for identifying heterozygous or homozygous female and male mammals carrying the mutated GDF-9B and/or GDF-9 genes. This knowledge of the biological function of the genes and their mutations can also be utilised to increase or decrease the ovulation rate of female mammals, or to induce sterility or reduced fertility in female mammals. In particular, an increase in ovulation rate in mammals may be induced by mimicking the heterozygous state, e.g. by reducing the biological activity of GDF-9 and/or GDF-9B by aound 50%. This can be achieved by a partial or short-term active immunisation regime whereby wild-type GDF-9 and/or GDF-9B or a functional variant or fragment thereof is administered to raise sufficient antibodies to partially neutralise endogenous GDF-9 and/or GDF-9B. Alternatively, said antibodies may be administered directly in a partial or short term passive immunisation regime.

A decrease in ovulation rate sufficient to reduce fertility or induce sterility may be induced by mimicking the homozygous state, e.g. by reducing the biological activity of GDF-9 and/or GDF-9B to around zero. This can be achieved by a full or long term

active immunisation regime whereby wild-type GDF-9 and/or GDF-9B or a functional variant or fragment thereof is administered to raise sufficient antibodies to affectively neutralise all of the endogenous GDF-9 and/or GDF-9B. Alternatively, said antibodies may be administered directly in a full or long term passive immunisation regime. Where the effect is permanent, sterility in the mammal is induced. Where the effect is reversible or temporary, a contraceptive effect is induced.

While the invention is broadly defined above, it will be appreciated by those persons skilled in the art that it is not limited thereto and includes embodiments of which the following descriptions give examples.

BRIEF DESCRIPTION OF DRAWINGS

Further aspects of the present invention will become apparent from the following description which is given by way of example only and with reference to the accompanying drawings in which:

Figure 1a shows the predicted amino acid sequence of sheep GDF9 protein. Numbers at the start of each line indicate amino acid positions of full-length unprocessed protein. Numbers in brackets indicate amino acid positions of the mature peptide. The RRHR furin protease cleavage site and predicted start of the mature processed peptide is shaded in grey. The filled triangle indicates the position of the single intron within the gene. The open triangles indicate positions of mutations that confer amino acid substitutions but are not associated with the sterility phenotype. The position of the [787] mutation associated with sterility is shaded black;

Figure 1b shows nucleotide substitution of four GDF-9 sequence mutations which result in an amino acid change compared to the wild-type sequence in Irish Cambridge and F700 Belclare sheep;

Figure 2a shows the predicted amino acid sequence of sheep GDF9B protein. Numbers at the start of each line indicate amino acid positions of full-length unprocessed protein. Numbers in brackets indicate amino acid positions of the mature peptide. The RRAR furin protease cleavage site and predicted start of the mature processed peptide is shaded in grey. The filled triangle indicates the position of the single intron within the gene. The open triangle indicates the position of a single Leu deletion polymorphism. The position of the [S1] and [S2] mutations associated with sterility are shaded black;

Figure 2b shows nucleotide substitutions of two GDF-9B sequence mutations which result in an amino acid change compared to the wild-type sequence in Irish Cambridge and F700 Belclare sheep;

Figure 3a shows a table representing the genotype and phenotype of sire R830 mated to three ewes 9704, 8783 and 7810, and their six female offspring. The phenotype of each animal is indicated as sterile S, fertile F or n/a (not applicable) for the male. The genotype of each animal is shown as ++ (wild type female for X-linked [S1] and [S2] mutations, wild type male and female [787] autosomal mutation), +Y (wild type male for X-linked [S1] or [S2]). Double copy carriers of the mutation are T/T, S1/S1 or S2/S2. Single copy carriers are T/+, S1/+ or S2/+. Sire R830 is hemizygous for X-linked [S2] as he can only carry one copy;

Figure 3b shows a table representing the genotypes and phenotypes within a F700 Cambridge pedigree. The pedigree represents sire 962101 mated to two ewes 962158 and 976234, and their four female offspring, and sire 930142 mated to ewe 8874 and their one female offspring. The phenotype of each animal is indicated as sterile S, fertile F or n/a (not applicable) for the male. The genotype of each animal is shown as ++ (wild type female for X-linked [S1] and [S2] mutations, wild type male and female for [787] autosomal mutations), +Y (wild type male for X-linked [S1] or [S2]. Double copy carriers of the mutations are T/T or S1/S1. Single copy carriers are T/+ or S1/+. Sires are hemizygous for X-linked [S1] as they can only carry one copy.

Figure 4 shows the nucleotide and amino acid sequences of wildtype sheep GDF-9 showing the positions of mutations in Irish Cambridge and F700 Belclare sheep. Numbers on the right indicate nucleotide position from the atg start codon. Numbers under each line indicate amino acid residue numbering from the start of the mature processed active peptide. Negative numbers indicate amino acids in the pro-region of the protein. The position of the single intron is marked by white boxes inserted into the sequence. Positions of the eight nucleotide polymorphisms are marked in bold within boxes, and named according to Table 1 using square brackets [ ]. The amino acids residues which are changed by the nucleotide polymorphisms are underlined. The taa stop codon indicates the end of the protein;

Figure 5 shows the nucleotide and amino acid sequences of sheep GDF-9B showing the positions of mutations in Irish Cambridge and F700 Belclare sheep. Numbers on the right indicate nucleotide position from the atg start codon. Numbers under each line indicate amino acid residue numbering from the start of the mature processed active peptide.

Negative numbers indicate amino acids in the pro-region of the protein. The position of the single intron is marked by a dashed inserted into the sequence. Positions of the four nucleotide polymorphisms are marked in bold within boxes, and named according to Table 1 using square brackets [ ]. The amino acids residues which are changed by the nucleotide polymorphisms are underlined. Asterisks (***) indicate the positions of the previously reported Hanna (Gln to Stop codon) and Inverdale (Val to Asp codon) mutations. The tga stop codon indicates the end of the protein;

Figure 6 shows the alignment of GDF-9 and GDF-9B protein sequence with other members of the TGFß superfamily members for which structures have been determined. The furin processing site is indicated as a solid gray block at the start of the sequences. The mature processed protein begins at amino acid residue position 4. Conserved cysteine molecules involved in disulphide bonds are shown in grey shading. Numbers along the bottom provide a relative reference to amino acid position, but do not represent the real amino acid residue number of each protein because gaps have been introduce to allow alignment of conserved protein regions. The asterisk * indicates the conserved cysteine that is present in most other TGFß family members except GDF9 and GDF9B, and which is responsible for the interchain disulphide bond present in most dimers. Boxed letters indicate the [787] serine (S) in GDF9 which is changed to phenylalanine in the mutants (position 86 on this diagram), and the [S2] serine (S) in GDF9B which is changed to isoleucine (position 118 in this diagram);

Figure 7 shows examples of the pattern of progesterone concentrations in plasma of actively immunized ewes. Antigen used for immunization and the ewe identification numbers are shown at the top of each graph. Markings by

vasectomized rams are indicated with arrows. Day 0 = corresponds to the beginning of thrice weekly sampling period; and

Figure 8 shows the average concentrations of progesterone in plasma following synchronization of luteal regression. Ewes were administered 100 ml of KLH, GDF9 peptide or BMP15 peptide antiplasma i.v. 4 days before synchronization with Estrumate (i.e. $PGF_{2\alpha}$, arrowed).

DETAILED DESCRIPTION OF INVENTION

The present invention is directed to new naturally-occurring mutations in the sheep GDF-9 and GDF-9B genes. It is shown for the first time that mutation of the GDF-9 gene causes increased ovulation rate as well as infertility in a manner similar to inactivating mutations in GDF-9B, and that GDF-9 is also essential for maintaining normal ovarian folliculogenesis in sheep. Furthermore, it is shown for the first time in any species, that sheep which are heterozygous for both GDF-9 and GDF-9B mutations have higher ovulation rates than sheep that are heterozygous for GDF-9 or GDF-9B mutations alone. These observations are supported by genotype, phenotype and immunisation data discussed below.

According to a first aspect of the present invention there is provided an isolated mutated GDF-9 nucleic acid molecule comprising a nucleotide sequence selected from the group consisting of:

a) SEQ ID NOs. 1, 3 or 5;

b) a sequence complementary to the molecule defined in a);

c) a functional fragment or variant of the sequences in a) or b);

d) an anti-sense sequence to any of the molecules defined in a), b) or c).

The present invention also provides an isolated mutated GDF-9 polypeptide comprising an amino acid sequence selected from the group consisting of SEQ ID NOs. 2, 4 or 6, or a functional fragment or variant thereof.

Whilst eight polymorphisms were located in the GDF-9 nucleotide sequence of the Belclare and Cambridge genomic DNA, only four resulted in amino acid substitutions in the corresponding polypeptide. However, of these, only one amino acid substitution was considered likely to change the function of the encoded polypeptide and therefore be involved in the observed changes in fertility rate seen in these sheep. This [787] serine to phenylalanine substitution at residue 395 replaces an uncharged polar group with a non-polar group at residue 77 of the mature coding region. Figures 1a, 1b, and 4, and Table 1 illustrate these nucleotide and amino acid changes.

Cambridge and Belclare ewes which were genotyped and found to be heterozygous for the GDF-9 [787] mutation were associated with a significant increase in ovulation rate over control ewes having none of the observed mutations (Table 3).

Cambridge and Belclare ewes which were genotyped and found to be homozygous for the GDF-9 [787] mutation were sterile.

The discovery of an inactivating mutation in GDF-9 associated with infertility and increased ovulation rate in sheep is the first evidence that GDF-9 is important for increasing ovulation rate. Although a knockout mutation of GDF-9 in mice has been shown to cause infertility, no effects for GDF-9 on increasing ovulation rate have been described. The present invention shows that small pertubations of protein structure within the GDF-9 mature peptide have severe consequences on protein activity.

The present invention also provides an isolated mutated GDF-9B nucleic acid molecule comprising a nucleotide sequence selected from the group consisting of:

a) SEQ ID NOs. 7, 9, 11, 13, 15 or 17;

b) a sequence complementary to the molecule defined in a)

c) an anti-sense sequence to any of the molecules defined in a) or b).

The present invention further provides an isolated mutated GDF-9B polypeptide comprising an amino acid sequence selected from the group consisting of SEQ ID NOs. 8, 10, 12, 14, 16 or 18.

Four polymorphisms were located in the GDF-9B nucleotide sequence of the Belclare and Cambridge genomic DNA. Two of these polymorphisms resulted in amino acid substitutions in the corresponding polypeptide sequence which were considered to change the polypeptide function and be involved in the observed changes in fertility rates seen in these sheep. The [S1] polymorphism resulted in C to T change at nucleotide 718 and introduced a premature stop codon (TAG) in the place of glutamic acid (Q, CAG) at amino acid residue 239 which presumably resulted in a complete loss of GDF-9B function. The [S2] polymorphism resulted in a serine to isolucine substitution at residue 367 of the unprocessed protein replacing an uncharged polar group with a non polar group. Figures 2 and 5 and Table 1 illustrate these nucleotide and amino acid changes.

Cambridge and Belclare ewes which were genotyped and found to be heterozygous for the GDF-9B [S1] or [S2] mutation were associated with a significant increase in ovulation rate over control ewes having none of the observed mutations (Table 3).

Cambridge and Belclare ewes which were genotyped and found to be homozygous for the GDF-9B [S1] or [S2] mutations were sterile.

These two new functional mutations in GDF-9B support the findings of previous studies, that mutations in this gene in sheep are associated with fertility control

(Galloway *et al.*, 2000). Inactivating mutations in GDF-9B cause increased ovulation rate and infertility in a dosage dependent manner. The serine to isoleucine change in the [S2] mutation and premature stop codon in the [S1] mutation, support the notion that perturbations of protein structure within the GDF-9B mature peptide have serious consequences in protein activity.

Surprisingly, ewes which are heterozygous for both of the GDF-9 [787] mutation and one of the GDF-9B [S1] or [S2] mutations have an even higher ovulation rate than animals that are heterozygous for a GDF-9 or GDF-9B mutation alone. The effects of the combination of GDF-9 [787] and GDF-9B [S1] mutations in Cambridge sheep and the combination of GDF-9 [787] and GDF-9B [S2] mutation in Belclare sheep appears to be additive (Table 3).

Thus, it is contemplated that an immunisation regime which could mimic these genotypes would be useful in modulating ovulation in female mammals. For example, a regime which would reduce the activity of endogenous GDF-9B and/or GDF-9 to about one half (as in heterozygous animals whereby only 50% of active molecules are expressed) could be used to increase ovulation and enhance fertility in female mammals. Conversely, an immunisation regime which would reduce the activity of endogenous GDF-9 and/or GDF-9B to approximately zero (as in homozygous animals where no active molecules are expressed) could be used to induce sterility.

The present invention further provides an isolated GDF-9 nucleic acid molecule comprising a mutation in at least one codon associated with receptor binding and/or dimerisation. The mutation preferably results in an amino acid substitution in the polypeptide encoded by the nucleic acid molecule, and said amino acid substitution is preferably present in the receptor binding domain and causes a disruption in receptor

binding. Alternatively, the amino acid substitution may be present in the dimersation domain to cause a disruption in dimerisation.

The invention further provides an isolated GDF-9B nucleic acid molecule comprising a mutation in at least one codon associated with receptor binding and/or dimerisation. The mutation preferably results in an amino acid substitution in the polypeptide encoded by the nucleic acid molecule, and said amino acid substitution is preferably present in the receptor binding domain and causes a disruption in receptor binding. Alternatively, the amino acid substitution may be present in the dimerisation domain to cause disruption in dimerisation.

Suitable programs for ascertaining the structure of polypeptides from the amino acid sequence which can used to determine the regions of the nucleotide sequence associated with dimerisation and/or receptor binding will be known to persons skilled in the art. Examples of suitable computer programs include The Modeller by Rockerfeller University and The SWISS Model developed by Swiss Protein database.

The mutations seen in the GDF-9 and GDF-9B genes which are associated with changes in fertility in the Cambridge and Belclare breeds have been shown to be associated with alterations in the function of the encoded polypeptides due to amino acid substitutions. Comparison between the location of these amino acid substitutions, with mutations, in other closely related TGF-ß molecules support the hypothesis that the biological activity of GDF-9 [787] is abolished due to a disruption in dimerisation whilst the GDF-9B [S2] mutation may abolish biological activity by disrupting receptor binding.

It is anticipated that other amino acid changes in the receptor-binding and dimerisation domains, or regions of the protein that disrupt protein folding of the mature peptide will have similar effects as would be appreciated by a skilled person and are included within the scope of the present invention.

The present invention also provides a method of identifying a mammal which carries a mutated nucleic acid molecule encoding GDF-9B, said method comprising the steps of:

i) obtaining a tissue or blood sample from the mammal;

ii) isolating DNA from the sample; and optionally

iii) isolating GDF-9B DNA from the DNA obtained at step i) or ii);

iv) probing said DNA with a probe complementary to either strand of the mutated GDF-9B DNA of SEQ ID NOs 11 or 17;

v) amplifying the amount of mutated GDF-9B DNA;

vi) determining whether the GDF-9B sequence DNA obtained in step v) carries a mutation associated with sterility or increased ovulation.

The present invention further provides a method of identifying a mammal which carries a mutated nucleic acid molecule encoding GDF-9, said method comprising the steps of:

i) obtaining a tissue or blood sample from the mammal;

ii) isolating DNA from the sample; and optionally

iii) isolating GDF-9 DNA from the DNA obtained at step i) or ii);

iv) probing said DNA with a probe complementary to either strand of the mutated GDF-9 DNA of SEQ ID NO 5;

v) amplifying the amount of mutated GDF-9 DNA;

vi) determining whether the GDF-9 sequence DNA obtained in step v) carries a mutation associated with sterility or increased ovulation.

The probe and primers that can be used in this method also forms a part of this invention. Said probes and primers may comprise a fragment of the nucleic acid molecule of the invention capable of hybridising under stringent conditions to a mutated GDF-9 or GDF-9B gene sequence. Such probes and primers are also useful, in studying the structure and function of the mutated genes, and for obtaining homologues of the genes from mammals other than sheep expressing the Cambridge and Belclare phenotypes.

Nucleic acid probes and primers can be prepared based on nucleic acids according to the present invention or sequences complementary thereto. A "probe" comprises a single stranded nucleic acid molecule having a known sequence which is attached to a detectable label or reporter molecule. Typical labels include radioactive isotopes, ligands, chemiluminescent agents, and enzymes. Such probes are used to locate and mark target DNA or RNA sequence by hybridizing to it.

A "primer" is a short nucleic acid, preferably DNA, 15 or more nucleotides in length, which are annealed to a complementary target DNA strand by nucleic acid hybridization to form a hybrid between the primer and the target DNA strand, then extended along the target DNA strand by a polymerase, preferably a DNA polymerase. Primer pairs can be used for amplification of a nucleic acid sequence, eg by the polymerase chain reaction (PCR) or other nucleic acid amplification methods well known in the art. PCR-primer pairs can be derived from the sequence of a nucleic acid according to the present invention, for example, by using computer programs intended for that purpose such as Primer (Version 0.5© 1991, Whitehead Institute for Biomedical Research, Cambridge, MA).

Methods for preparing and using probes and primers are described, for example, in Sambrook et al. Molecular Cloning: A Laboratory Manual, 2nd ed, vol. 1-3, ed Sambrook et al. Cold Spring Harbour Laboratory Press, Cold Spring Harbour, NY, 1989.

Probes or primers can be free in solution or covalently or noncovalently attached to a solid support by standard means.

For the amplification of a target nucleic acid sequence (eg by PCR) using a particular amplification primer pair, stringent conditions are conditions that permit the primer pair to hybridise only to the target nucleic acid sequence to which a primer having the corresponding wild type sequence (or its complement) would bind.

Nucleic acid hybridization is affected by such conditions as salt concentration, temperature, or organic solvents, in addition to the base composition, length of the complementary strands, and the number of nucleotide base mismatches between the hybridising nucleic acids, as will be readily appreciated by those skilled in the art.

When referring to a probe or primer, the term "specific for (a target sequence)" indicates that the probe or primer hybridises under stringent conditions only to the target sequence in a given sample comprising the target sequence. Commonly, stringent hybridization conditions are 6 x SSC at 55°C.

In another embodiment, the present invention provides the use of a nucleic acid molecule which is complementary to either strand of the mutated DNA of SEQ ID NOs. 11 or 17 as a marker to identify a mammal carrying a mutated nucleic acid molecule encoding GDF-9B.

The term 'either strand' refers to both the strand of DNA shown in the Sequence ID Number that is being referred to or its complementary strand which is not shown in the sequence listing but which can be determined therefrom.

In a further embodiment, the present invention provides the use of a marker as described above in a method of DNA marker assisted selection of mammals carrying mutated GDF-9B associated with either enhanced ovulation or sterility.

In another embodiment, the present invention provides the use of a nucleic acid molecule which is complementary to either strand of the mutated DNA of SEQ ID NO 5

as a marker to identify a mammal carrying a mutated nucleic acid molecule encoding GDF-9.

The present invention further provides the use of a marker as described above in a method of DNA assisted selection of mammals carrying mutated GDF-9 either enhanced ovulation or sterility.

The above markers and methods of marker assisted selection are useful to identify sequence variants in individual animals that are associated with increased ovulation of that animal. Although these variants may not necessarily give rise to the increased ovulation or sterility trait directly, they will be sufficiently closely associated with it to predict the trait. The methods by which these sequence variants are identified are known in the art, and include, but are not limited to, restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism AFLP, direct sequencing of DNA within or associated with the GDF-9 gene, or identification and characterisation of variable number of tandem repeats (VNTR), also known as microsatellite polymorphisms. Thus, the genetic marker may have utility in DNA selection of animals having increased ovulation.

In a further embodiment, the present invention provides a construct or vector comprising a nucleic acid molecule substantially as described above.

The term "construct" as used herein refers to an artificially assembled or isolated nucleic acid molecule which includes the gene of interest. In general a construct may include the gene or genes of interest and appropriate regulatory sequences. It should be appreciated that the inclusion of regulatory sequences in a construct is optional for example, such sequences may not be required in situations where the regulatory sequences of a host cell are to be used. The term construct includes vectors but should not be seen as being limited thereto.

The term "vector" as used herein encompasses both cloning and expression vectors. Vectors are often recombinant molecules containing nucleic acid molecules from several sources.

A "cloning vector" refers to a nucleic acid molecule originating or derived from a virus, a plasmid or a cell of a higher organism into which another exogenous (foreign) nucleic acid molecule of interest, of appropriate size can be integrated without loss of the vector's capacity for self-replication. Thus vectors can be used to introduce at least one foreign nucleic acid molecule of interest (e.g. gene of interest) into host cells, where the gene can be reproduced in large quantities.

An "expression vector" refers to a cloning vector which also contains the necessary regulatory sequences to allow for transcription and translation of the integrated gene of interest, so that the gene product of the gene can be expressed.

The cloning vector may be selected according to the host or host cell to be used. Useful vectors will generally have the following characteristics:

(a) the ability to self-replicate;

(b) the possession of a single target for any particular restriction endonuclease; and

(c) desirably, carry genes for a readily selectable marker such as antibiotic resistance.

Two major types of vector possessing these characteristics are plasmids and bacterial viruses (bacteriophages or phages). Presently preferred vectors are bacterial, insect or mammalian vectors and may include the following: the pUC, pBlueScript, pGEM, PGEX, pBK-CMV, lambda ZAP, lambda GEM, pEFIRES-P, pUB6/V5/His, pBC1, pADTrack-CMV, pAdenovator, pAdEasy-1, pSFV-PD, pCA3, pBABE, pPIC9,

pA0815, pET and pSP series. However, this list should not be seen as limiting the scope of the present invention.

Examples of preferred expression systems are as follows:

1. For an *in vitro* cell expression system, the 293T cell system with a pEFIRES-P vector (Hobbs S *et al.*,1998, Biochem Biophys Res Commun. 252: 368-72) which confers puromycin resistance may be used. For coexpression of two genes, the aforementioned vector may be modified to change the antibiotic resistance gene to bleomycin resistance. Alternatively, the co-expression of two genes and the selection gene can be achieved by constructing a tricistronic expression vector. A corresponding stably transfected insect cell system can also be used, e.g. the S2 cell system using "DES" vector expression system; www.invitrogen.com.

2. With respect to expressing GDF's in all tissues of transgenic animals, one approach is to use the pUB6/V5-His A vector (www.invitrogen.com) to make the constructs. For tissue-specific expression the rat PEPCK 0.6 kb promoter for liver and kidney expression can be included in the construct by replacing the Ubi-C promoter in the pUB6/V5-His A vector with the PEPCK promoter. For GDF expression in mammary tissue another promoter system would be preferred. For this tissue one approach would be to use the bovine β-lactoglobulin gene promoter and/or the bovine α S1 casein promoter (e.g. pBC1 vector, www.invitrogen.com) to drive the expression of the GDFs into milk. For global over-expression in transgenic animals, the CMV enhanced β-actin

promoter (Okabe M, et al.; FEBS Letters 407: 313-319, 1997) or a modified EF1 α-promoter can be used also (Taboit-Dameron F, et al., Transgenic Research 8: 223-235, 1998).

Adenoviruses, retroviruses and alphaviruses are other suitable mammalian expression systems. A typical approach to those skilled in the art is that described by (TC He *et al.*, 1998, Proc Natl Acad Sci USA. 95: 2509-14). With respect to GDF expression the pAd Track-CMV vector or pAdenovator vectors (www.qbiogene.com) can be used to make the construct which is then co-transformed with pAd Easy-1 adenoviral plasmid into *E. coli* to generate a recombinant adenoviral genome which contains a CMV-promoter driven GDF expression cassette. This recombinant adenoviral genome is then transfected into 293T cells to make the virus stock. Alternative methods for generating adenoviruses can also be used for the same purpose (e.g. PCA3 plasmid based gene transfer (www.microbix.com); or COS-TPC method (Miyake S *et al.*, 1996, Proc Natl Acad Sci USA. 93: 1320-4).

3. Non-cytopathogenic Semliki Forest viruses expressing GDF's can be generated using, for example, pSFV-PD vectors as described by Lundstrom et al., Histochem Cell Biol 115: 83-91, 2001. Furthermore, retroviral expression systems based on, for example, pBABE vectors, can be used for expressing GDF's in mammalian cells (Morgenstern, JP and Land, H, 1990; Nucleic Acids Res 18: 3587-3596).

4. Yeast cells (e.g. *Pichia pastoris*, *Saccharomyces cerevisiae*) are another well established expression system to those skilled in the art (MA Romanos *et al.*,

1992, Yeast. 8: 423-88). For example, the pPIC9 vector (www.invitrogen.com) can be used in *Pichia pastoris* for the expression of GDF's. For coexpression of two genes, the vector pA0815 (www.invitrogen.com) is a preferred candidate.

5. *Echerichia coli* (*E. coli*) is a standard laboratory expression system in widespread use. For example, the pET expression system (www.novagen.com) can be used to express recombinant mammalian GDF-9 and GDF-9B

The DNA molecules of the invention may be expressed by placing them in operable linkage with suitable control sequences in a replicable expression vector. Control sequences may include origins of replication, a promoter, enhancer and transcriptional terminator sequences amongst others. The selection of the control sequence to be included in the expression vector is dependent on the type of host or host cell intended to be used for expressing the DNA as would be understood by a person skilled in the art.

The term "operably linked" or grammatical variant thereof as used herein means that the regulatory sequences necessary for expression of the gene of interest are placed in the nucleic acid molecule in the appropriate positions relative to the gene to enable expression of the gene.

As used herein the term "regulatory sequences" refers to certain nucleic acid sequences such as origins of replication, promoters, enhancers, polyadenylation signals, terminators and the like, that enable expression of the nucleic acid molecule of interest.

The term "expression" as used herein broadly refers to the process by which a nucleic acid molecule is converted by transcription and then translation into a protein.

The term "gene" as used herein refers to a nucleic acid molecule comprising an ordered series of nucleotides that encodes a gene product (i.e. specific protein). The expression vectors useful in the present invention may contain at least one expression control sequence that is operatively linked to the DNA sequence or fragment to be expressed. The control sequence is inserted in the vector in order to control and to regulate the expression of the cloned DNA sequence. Examples of useful expression control sequences are the lac system, the trp system, the tac system, the trc system, major operator and promoter regions of phage lambda, the glycolytic promoters of yeast acid phosphatase, e.g. Pho5, the promoters of the yeast alpha-mating factors, and promoters derived from polyoma, adenovirus, retrovirus, and simian virus, and cytomegalovirus e.g. the early and late promoters of SV40, and other sequences known to control the expression of genes of prokaryotic and eukaryotic cells and their viruses or combinations thereof.

In the construction of a vector it is also an advantage to be able to identify the bacterial clone carrying the vector incorporating the foreign DNA. Such assays include measurable colour changes, antibiotic resistance and the like. In one preferred vector, the β-galactosidase gene is used, in which clones are detectable as blue or white phenotypes on X-gal plates. This facilitates selection. Once selected, the vectors may be isolated from the culture using standard procedures.

Depending on the host used, transformation and transfection is performed according to standard techniques appropriate to such cells. For prokaryotes or other cells that contain substantial cell walls, the calcium treatment process (Cohen, S N Proceedings, National Academy of Science, USA 69 2110 (1972)) may be employed. For mammalian cells without such cell walls, the calcium phosphate precipitation method of Graeme and Van Der Eb, Virology 52:546 (1978) or liposomal reagents are preferred.

Upon transformation of the selected host with an appropriate vector the polypeptide encoded can be produced, often in the form of a fusion protein, by culturing the host cells. The polypeptide of the invention may be detected by rapid assays as indicated above. The polypeptide is then recovered and purified as necessary. Recovery and purification can be achieved using any procedures known in the art, for example by absorption onto and elution from an anion exchange resin. This method of producing a polypeptide of the invention constitutes a further aspect of the present invention.

Host cells transformed with the vectors or constructs of the invention also form a further aspect of the present invention.

The term "host cell" refers to a cell which is capable of containing a vector or construct and supports the replication and/or expression of the vector or construct. Suitable hosts cells may include *E.coli*, yeast or mammalian cells but should not be limited thereto.

The present invention also provides a cell line comprising a host cell substantially as described above.

Knowledge of the mutated gene sequences can be applied to a test for identifying heterozygous or homozygous female and male mammals carrying the mutated GDF-9B and/or GDF-9 genes. This knowledge of the biological function of the genes and their mutations can also be utilised to increase or decrease the ovulation rate of female mammals, or to induce sterility or reduced fertility in female mammals. In particular, an increase in ovulation rate in mammals may be induced by mimicking the heterozygous state whereby only half of the 'normal' amount of GDF-9 and/or GDF-9B is expressed. This can be achieved by a partial or short-term active regime whereby wild-type GDF-9 and/or GDF-9B or a functional variant or fragment thereof is administered to raise sufficient antibodies to partially neutralise endogenous GDF-9 and/or GDF-9B.

Alternatively, said antibodies may be administered directly in a partial or short term passive immunisation regime.

A decrease in ovulation rate sufficient to reduce fertility or induce sterility may be induced by mimicking the homozygous state, whereby no active GDF-9 and/or GDF-9B is expressed. This can be achieved by a full or long term active immunisation regime whereby wild-type GDF-9 and/or GDF-9B or a functional variant or fragment thereof is administered to raise sufficient antibodies to affectively neutralise all of the endogenous GDF-9 and/or GDF-9B. Alternatively, said antibodies may be administered directly in a full or long term passive immunisation regime. Where the effect is permanent, sterility in the mammal is induced. Where the effect is reversible or temporary, a contraceptive effect is induced.

Thus in a further aspect, the present invention provides a method altering GDF-9 and/or GDF-9B bioactivity in a female mammal so as to modulate ovulation comprising the steps of either:

(a) inducing a partial immunisation response to endogenous GDF-9 and/or GDF-9B to partially reduce bioactivity thereof and enhance ovulation; or

(b) inducing a full immunisation response to endogenous GDF-9 and/or GDF-9B to substantially reduce bioactivity thereof and induce sterility.

The immunisation response may be induced by administration of an antigenic composition comprising:

i) a GDF-9 polypeptide or a functional fragment or variant of GDF9; and/or

ii) a GDF-9B polypeptide or a functional fragment or variant of GDF-9B;

together with a pharmaceutically or veterinarily acceptable carrier and/or diluent to a mammal in need thereof.

The antigenic composition may include an adjuvant to induce a partial immunisation response and enhance ovulation.

An example of such an adjuvant includes DEAE-Dextran adjuvant.

Adjuvants such as DEAE-Dextran are selected so as to provoke a partial immune response upon administration to a mammal.

The antigenic composition may comprise an alternative adjuvant to induce a strong immunisation response and reduce fertility or induce sterility.

Examples of such an adjuvant include Freunds adjuvant.

Adjuvants such as Freunds are selected so as to provoke a strong immune response upon administration to a mammal.

Partial or strong immunisation may be induced either actively by the administration of the aforementioned antigenic compositions, or passively, by administration of antibodies raised against said antigenic composition or anti-sera comprising said antibodies.

Partial immunisation may also be induced by a short term administration regime, whereby the antigenic composition or antibodies thereto are administered over a short time period such as one to two months.

Full immunisation may be induced by a long term administration regime, whereby the antigenic composition or antibodies therein are administered over a long time period such as six months or more.

The type of adjuvant, antigenic composition, and administration regime are selected to induce the desired response as would be understood by a skilled worker. A particularly desired response is an enhanced ovulation rate and associated increased fertility. Such a method is useful to enhance the reproductive efficiencies and/or enhance multiple ovulations from high value mammals.

A further desired response is a decrease in ovulation rate and associated reduced fertility. Preferably such a response is permanent and results in sterility of the female mammal. This method provides an alternative to surgical methods that are presently used to induce sterility and is much less invasive and carries no risk of infection etc as is the case with surgery.

When the response induced is temporary or reversible, the method may be used as a method of contraception. Again such a method provides advantages over the currently used methods of contraception (i.e. administration of progesterone and/or oestrogen) which are associated with health risks. In addition, the method of the present invention may be used to prevent ovulation in a female mammal, such as a race horse, greyhound etc, when the natural oestrous cycle would interfere with the performance of such a mammal. As such an effect is temporary or reversible, the mammal will not suffer any deleterious effects.

Such a temporary/reversible effect has been observed in sheep following administration of GDF-9B peptide over a period of time sufficient to inhibit ovulation, which upon cessation of administration, subsequently became pregnant.

The present invention further contemplates inducing a desired response by administration of the mutated GDF-9B polypeptide molecules of the invention. Although such polypeptide molecules are likely to be inactive as they comprise a mutation in the receptor binding and/or dimerisation domains, such polypeptides, when

administered in sufficient amounts, may compete with the endogenous GDF-9 and/or GDF-9B binding and/or dimerisation to reduce the biological activity thereof. Such a response, if it results in a partial reduction of endogenous GDF-9 and/or GDF-9B activity will result in enhanced ovulation and fertility. If such administration results in a full reduction of endogenous GDF-9 and/or GDF-9B activity, the response induced will be sterility.

Also encompassed by the present invention is a transgenic non-human mammal wherein one copy of the endogenous GDF-9 and/or GDF-9B gene has been knocked out. Such a mammal would have increased ovulation and enhanced fertility.

Such a transgenic mammal may be produced by known methods (Wells et al, 1998. Reprod Fertil Dev: 10:615-26; Clark 2002, Methods Mol Biol, 180: 273-87; Cousens et al 1994, Mol Reprod Dev. 39:384-91; Chen et al 2002, Biol Reprod. 67: 1488-92; Arat et al 2001, Mol Repord Dev. 60: 20-6) and may comprise the steps of introducing to the genetic material of the mammal at least one nucleic acid molecule comprising a nucleotide sequence selected from the group consisting of:

- SEQ ID NOs 1 or a functional fragment or variant thereof; and
- SEQ ID NOs 7 or 13 but not both; or
- SEQ ID NOs 3 or a functional fragment or variant thereof; and
- SEQ ID NOs 9 or 15 but not both, using a vector or construct according to the invention.

In a further aspect the present invention provides a method of modulating the ovulation rate of a female mammal comprising the steps of:

a) identifying the nucleotide sequences of GDF-9 or GDF-9B carried by the female mammal;

b) administering as appropriate having regard to the GDF-9 and/or GDF-9B genes present in the mammal, an effective amount of an agent selected from the group consisting of:

1) an immunising effective amount of a GDF-9 polypeptide and/or an immunising effective amount of a GDF-9B polypeptide substantially as described above;

2) antisense nucleic acid molecule(s) directed towards nucleic acid(s) encoding:

i) a GDF-9 polypeptide substantially as described above; and/or

ii) a GDF-9B polypeptide substantially as described above.

In a further embodiment, the present invention provides a method for breeding a mammal having increased ovulation comprising the steps of:

a) identifying the nucleotide sequences of GDF-9 or GDF-9B carried by the female mammal it is proposed to breed from;

b) identifying the nucleotide sequences of GDF-9 or GDF-9B carried by the male mammal it is proposed to breed from;

c) selecting the female and male animals that will result in progeny having the following characteristics:

i) a single copy of a mutated GDF-9 nucleotide sequence comprising:

A) SEQ ID NO 5; or

B) a functional variant or fragment of the molecule in A); or

C) a sequence complementary to the molecule in A) or B); and/or

ii) a single copy of mutated GDF-9B nucleotide sequence comprising:

A) SEQ ID NOs 11 or 17; or

B) a sequence complementary to the molecule(s) in A).

The mammals selected for breeding according to the method described above may result in progeny having the following characteristics:

i) a single copy of a mutated GDF-9 nucleotide sequence comprising:

A) SEQ ID NO 5; or

B) a functional variant or fragment of the molecule in A); or

C) a sequence complementary to the molecule in A) or B);

ii) a single copy of a mutated GDF-9B nucleotide sequence comprising:

A) SEQ ID NOs 11 or 17; or

B) a sequence complementary to the molecule(s) in A).

In a still further embodiment, the present invention provides a method for selecting a female mammal for breeding on the basis of possessing an increased rate of ovulation comprising the steps of identifying a female mammal possessing only a single mutated copy of:

1) a mutated GDF-9 nucleotide sequence comprising:

a) SEQ ID NO 5; or

b) a functional variant of the molecule of a); or

c) a sequence complementary to the molecules in a) or b);

and/or

2) a mutated GDF-9B nucleotide sequence comprising:

a) SEQ ID NOs 11 or 17; or

b) a sequence complementary to the molecules in a).

Preferably the mammal selected has both a single mutated copy of GDF-9 and GDF-9B.

In a further embodiment, the present invention provides a composition comprising:

i) a mutated GDF-9 polypeptide comprising an amino acid sequence selected from the group consisting of:

A) SEQ ID NOs. 2, 4 or 6; or

B) a functional fragment or variant of the sequences in A); and/or

ii) a mutated GDF-9B polypeptide comprising an amino acid sequence selected from the group consisting of SEQ ID NOs. 8, 10,12,14,16 or 18

together with a pharmaceutically or veterinarily acceptable carrier and/or diluent.

The preparation of pharmaceutical compositions including pharmaceutical carriers are well known in the art, and are set out in textbooks such as Remington's Pharmaceutical Sciences, 19th Edition, Mack Publishing Company, Easton, Pennsylvania, USA.

The compounds and compositions of the invention may be administered by any suitable route, and the person skilled in the art will readily be able to determine the most suitable route and dose for the condition to be treated. Dosage will be at the discretion of the attendant physician or veterinarian, and will depend on the nature and state of the condition to be treated, the age and general state of health of the subject to be treated, the route of administration, and any previous treatment which may have been administered.

The carrier or diluent, and other excipients, will depend on the route of administration, and again the person skilled in the art will readily be able to determine the most suitable formulation for each particular case.

As mentioned above, the polypeptide of the present invention may be administered directly to a female mammal.

The term "protein, or polypeptide" as used herein refers to a protein encoded by the nucleic acid molecule of the invention, including fragments, mutations and homologues having the same biological activity i.e. ovulation modulating activity. The protein or polypeptide of the invention can be isolated from a natural source, produced by the expression of a recombinant nucleic acid molecule, or chemically synthesized.

It is to be clearly understood that the invention also encompasses peptide analogues, which include but are not limited to the following:

1. Compounds in which one or more amino acids is replaced by its corresponding D-amino acid. The skilled person will be aware that retro-inverso amino acid sequences can be synthesised by standard methods; see for example Choreo and Goodman, 1993;

2. Peptidomimetic compounds, in which the peptide bond is replaced by a structure more resistant to metabolic degradation. See for example Olson et al, 1993; and

3. Compounds in which individual amino acids are replaced by analogous structures for example, gem-diaminoalkyl groups or alkylmalonyl groups, with or without modified termini or alkyl, acyl or amine substitutions to modify their charge.

The use of such alternative structures can provide significantly longer half-life in the body, since they are more resistant to breakdown under physiological conditions.

Methods for combinatorial synthesis of peptide analogues and for screening of peptides and peptide analogues are well known in the art (see for example Gallop et al, 1997, Nature Biotechnology, 15: 328-330).

For the purposes of this specification, the term "peptide and peptide analogue" includes compounds made up of units which have an amino and carboxy terminus separated in a 1, 2, 1, 3, 1, 4 or larger substitution pattern. This includes the 20 naturally-occurring or "common" α-amino acids, in either the L or D configuration, the biosynthetically-available or "uncommon" amino acids not usually found in proteins, such as 4-hydroxyproline, 5-hydroxylysine, citrulline and ornithine; synthetically-derived α-amino acids, such as α-methylalanine, norleucine, norvaline, Cα- and *N*-alkylated amino acids, homocysteine, and homoserine; and many others as known in the art.

It also includes compounds that have an amine and carboxyl functional group separated in a 1,3 or larger substitution pattern, such as β-alanine, γ-amino butyric acid, Freidinger lactam the bicyclic dipeptide (BTD) (Freidinger *et al*, 1982, J. Org. Chem 59: 104-109; Nagai and Sato, 1985, Tetrahedron Lett. 26: 647-650), amino-methyl benzoic acid, and others well known in the art. Statine-like isosteres, hydroxyethylene isosteres, reduced amide bond isosteres, thioamide isosteres, urea isosteres, carbamate

isosteres, thioether isosteres, vinyl isosteres and other amide bond isosteres known to the art are also useful for the purposes of the invention.

A "common" amino acid is a L-amino acid selected from the group consisting of glycine, leucine, isoleucine, valine, alanine, phenylalanine, tyrosine, tryptophan, aspartate, asparagine, glutamate, glutamine, cysteine, methionine, arginine, lysine, proline, serine, threonine and histidine. These are referred to herein by their conventional three-letter or one-letter abbreviations.

An "uncommon" amino acid includes, but is not restricted to, one selected from the group consisting of D-amino acids, homo-amino acids, N-alkyl amino acids, dehydroamino acids, aromatic amino acids (other than phenylalanine, tyrosine and tryptophan), ortho-, meta- or para-aminobenzoic acid, ornithine, citrulline, norleucine, α-glutamic acid, aminobutyric acid (Abu), and α-α disubstituted amino acids.

In a further embodiment, the present invention provides a method of modifying the function of the corpus luteum by administering supplementary GDF-9 or GDF-9 B, or analogues thereof, or GDF-9 or GDF-9B antagonists to female mammals.

The present invention also encompasses ligands directed to the polypeptides of the invention.

The term "ligand" refers to any molecule which can bind to another molecule such as a polypeptides or peptide, and should be taken to include, but not be limited to, antibodies, cell surface receptors or phage display molecules.

It should be appreciated that the term "antibody" encompasses fragments or analogues of antibodies which retain the ability to bind to a polypeptide of the invention, including but not limited to Fr, F $(ab)_2$ fragments, ScFv molecules and the like. The antibody may

be polyclonal but is preferably monoclonal. In some embodiments the ligand may be a phage display molecule.

The invention also includes adenovirus-based gene therapy techniques for expressing GDF-9B and GDF-9/GDF-9B in cell cultures, organ cultures and whole experimental animals for manipulating ovarian follicular protein synthesis or production.

Definitions

Throughout this specification it should understood that the nucleic acid molecule may be a RNA, cRNA, genomic DNA or cDNA molecule, and may be single or double-stranded. The nucleic acid molecule may also optionally comprise one or more synthetic non-natural or altered nucleotide bases, or combinations thereof.

The term "analogues" above refers to a compound which has a biological function with improved characteristics over the native compounds (e.g. such an analogue may have a longer half-life than the native compound.)

The term "antagonist" refers to a compound which inhibits the effect of another compound. In this context, the antagonist could refer to a purified antibody, a sera or serum containing an antibody or a plasma or pool of plasma containing an antibody that would neutralise GDF-9 or GDF-9B.

The term "partial immunisation" refers to immunisation of an animal either active or passive of sufficient antigen/antibody to allow for instigation of an immune response to be mounted against the antigen; but the degree of antigen/antibody administered and/or the means of administration are such that insufficient antibodies are produced by the immunised animal to effectively neutralise all the antigen of interest.

The term "full immune response" refers to the immune response of animal which has been fully immunised i.e. the response mounted by the immunised animal results in production of sufficient antibodies to effectively neutralise all the antigen of interest.

The term "introducing" (or grammatical variations thereof) when used in the context of inserting a nucleic acid molecule into a cell, means "transfection" or "transformation" or "transduction" and includes reference to any method for incorporation or transfer of a nucleic acid molecule into a eukaryotic or prokaryotic cell for expression or replication thereof (for example this may include but should not be limited to insertion of a nucleic acid into: a chromosome, mitochondrial DNA, an autonomous replicon (eg. a plasmid).

The term "transduction" as used herein, refers to the process of transferring genetic information from a nucleic acid molecule from one cell to another by way of a viral vector. The term "transfection" as used herein, refers to the uptake, incorporation, and expression of recombinant DNA by eukaryotic cells.

The term "transformation" as used herein refers to a process by which the genetic material carried by an individual cell is altered by incorporation of exogenous DNA into its genome.

The term "variant" as used herein refers to nucleotide and polypeptide sequences wherein the nucleotide or amino acid sequence exhibits substantially 60% or greater homology with the nucleotide or amino acid sequence of the Figures, preferably 75% homology and most preferably 90-95% homology to the sequences of the present invention. – as assessed by GAP or BESTFIT (nucleotides and peptides), or BLASTP (peptides) or BLAST X (nucleotides). The variant may result from modification of the native nucleotide or amino acid sequence by such modifications as insertion, substitution or deletion of one or more nucleotides or amino acids or it may be a naturally-occurring variant. The term "variant" also includes homologous sequences

which hybridise to the sequences of the invention under standard hybridisation conditions defined as 2 x SSC at 65°C, or preferably under stringent hybridisation conditions defined as 6 x SSC at 55°C, provided that the variant is capable modulating the ovulation rate of a female mammal or altering ovarian function. Where such a variant is desired, the nucleotide sequence of the native DNA is altered appropriately. This alteration can be effected by synthesis of the DNA or by modification of the native DNA, for example, by site-specific or cassette mutagenesis. Preferably, where portions of cDNA or genomic DNA require sequence modifications, site-specific primer directed mutagenesis is employed, using techniques standard in the art.

A "fragment" of a nucleic acid is a portion of the nucleic acid that is less than full length, and comprises at least a minimum sequence capable of hybridizing specifically with a nucleic acid molecule according to the invention, or a sequence complementary thereto, under stringent conditions as defined below. A "fragment" of a polypeptide is a portion of the polypeptide which is less than full length, but which still retains the biological function of either; increasing or decreasing the ovulation rate of a mammal, causing sterility in a mammal; or altering the regulation of the corpus luteum. Hence, a fragment according to the invention has at least one of the biological activities of the nucleic acid or polypeptide of the invention. However, it will be appreciated that the biological activity of a fragment of the GDF-9 sequence of the present invention encompass only those mutations which will increase the ovulation rate in female mammals heterozygous for the mutation.

The term "isolated" means substantially separated or purified away from contaminating sequences in the cell or organism in which the nucleic acid naturally occurs and includes nucleic acids purified by standard purification techniques as well as nucleic acids prepared by recombinant technology, including PCR technology, and those chemically synthesised.

The term "modulation of ovulation" means increasing or decreasing the rate of ovulation compared to the endogenous rate observed in an untreated animal.

The term "hybridization" or grammatical variants thereof means the process of joining two complementary strands of DNA or one each of DNA and RNA to form a double stranded molecule.

When referring to a probe or primer, the term "specific for (a target sequence)" indicates that the probe or primer hybridizes under stringent conditions only to the target sequence in a given sample comprising the target sequence.

EXAMPLES

Non-limiting examples illustrating the invention will now be provided. It will be appreciated that the above description is provided by way of example only and variations in both the materials and techniques used which are known to those persons skilled in the art are contemplated.

Methodology

Animals

The flocks of Cambridge and Belclare sheep at The Sheep Research Centre of Teagasc, Athenry, were routinely examined for ovulation rate at the beginning of each mating season using laparoscopy. The examination was done once before joining and once after the first mating of the joining period. This data has been collected each year since these flocks were established. The flocks were self-contained with at least 5 males used for mating each year. In addition rams from these flocks were progeny tested for ovulation rate by crossing with Galway and Scottish Blackface ewes – both low prolificacy

breeds. Ovulation rate measurements were done by laparoscopy under licence from The Minister of Health under the Cruelty to Animals Act (1876) EU Directive 86/609/EC.

When sterile ewes were first detected they were checked for the possibility that they were freemartins but this could not be confirmed (Hanrahan, 1991). Blood samples were retained for DNA extraction from the sterile Cambridge ewes born in 1990 and later years and from essentially all of the F700 Belclare sterile females born since 1993. This material has been supplemented by blood samples for DNA extraction collected from fertile ewes in these flock from 1992 onwards.

Ovulation rate data were analysed by least squares procedures with the individual animal as the experimental unit using the GLM procedure of SAS. The factors in the models were ewe, age, year of record, and the number of copies (0 or 1) of each of the mutations described below.

Samples

Genomic DNA was isolated from Irish Cambridge and F700 Belclare sheep either from frozen stored buffy coat or directly from white blood cells in whole blood. Parentage of key pedigrees was verified with autosomal sheep microsatellite markers OarHH64 (sheep chromosome 4), OarCP34 (sheep chromosome 3) and OarFCB304 (sheep chromosome 19).

Sequencing and Mutation Detection

The sheep GDF-9 and GDF-9B genes were amplified using the polymerase chain reaction (PCR) with primers designed from published sheep sequences (sheep genomic GDF-9B exon 1, AF236078; sheep genomic GDF-9B exon 2, AF236079; sheep genomic GDF-9 exon 1 and 2, AF078545).

The PCR primers used were as follows:

GDF-9B exon 1	B13: 5'-ACTGCTGCCTTGTCCCAC-3'
	B28: 5'-AGGCAATGTGAAGCCTGACA-3'
GDF-9B exon 2	B25: 5'-CAGTTTGTACTGAGCAGGTC-3'
	O4: 5'-TTCTTGGGAAACCTGAGCTAGC-3'
GDF-9 exon 1	G1: 5'-GAATTGAACCTAGCCCACCCAC-3'
	G4: 5'-AGCCTACATCAACCCATGAGGC-3'
GDF-9 exon 2	G5: 5'-ATCCCACCCTGACGTTTAAGGC-3'
	G7: 5'-TCCTCCCAAAGGCATAGACAGG-3'

The resulting PCR products were sequenced on an ABI 373 sequencer.

Single Stranded Conformational Polymorphism Detection

SSCP (single stranded conformational polymorphism) was carried out on 9 Belclare rams involved in the progeny testing programme and on the half sib progeny of three of these rams (n = 58 (29, 17, and 12 progeny respectively)) and also on 2 Cambridge rams one of whom was progeny tested. In addition, seven purebred daughters of two of the Belclare rams were tested along with four of the five dams involved.

GDF-9B genotypes were determined by analysis of three nucleotide fragments which spanned most of exon 2. Fragments analysed by SSCP were:

353 bp, exon 2	primer 9B-359 5'-CGC TTT GCT CTT GTT CCC TCT-3'
	primer 9B-691 5'-CCT CAC TAC CTC TTG GCT GCT-3'

273 bp, exon 2	primer 9B-664 5'-GGG TTC TAC GAC TCC GCT TC-3'
	primer 9B-916 5'-GGT TAC TTT CAG GCC CAT CAT-3'
312 bp, exon 2	primer 9B-915 5'-CAT GAT GGG CCT GAA AGT AAC-3'
	primer 9B-1205 5'-GGC AAT CAT ACC CTC ATA CTC C-3'

Primers were designed from nucleotide sequence Genbank Accession number AF236079 and primer names correspond to nucleotide position within that sequence.

GDF-9 genotypes were determined by analysis of five fragments which spanned exon 1, part of the intron and most of exon 2. Fragments analysed by SSCP were:

462 bp, exon 1	primer G9-1734 5'-GAA GAC TGG TAT GGG GAA ATG-3'
	primer G9-2175 5'-CCA ATC TGC TCC TAC ACA CCT-3'
294 bp, intron	primer G9-2676 5'-GTG TGA GAG AGA TGG GAG CA-3'
	primer G9-2947 5'-AAG AGG AAA ACT ATC AAA AGA CA-3'
296 bp, exon 2	primer G9-3270: 5'-TGG CAT TAC TGT TGG ATT GTT TT-3'
	primer G9-3546: 5'-CAA GAG GAG CCG TCA CAT CA-3'
206 bp, exon 2	primer G9-3543: 5'-GAT TGA TGT GAC GGC TCC TCT-3'
	primer G9-3728: 5'-GGG AAT GCC ACC TGT GAA AAG-3'
221 bp, exon 2	primer G9-3939: 5'-TCT TTT TCC CCA GAA TGA ATG T-3'
	primer G9-4140: 5'-CAC AGG ATG GTC TTG GCA CT-3'

Primers were designed from nucleotide sequence Genbank Accession number AF078545 and primer names correspond to nucleotide position within that sequence.

Amplification was carried out for 30 cycles in a 40 µL reaction mixture, using 150 ng of genomic DNA, with 1.5 mM or 3 mM magnesium and an annealing temperature of 55 to 58° C. PCR fragments were analysed by SSCP in polyacrylamide gels with overnight migration at 9-15 V/cm, 15°C.

Single Nucleotide Polymorphism Detection Assays

The [E1] polymorphism identified in GDF-9 exon 1 produced a G to A nucleotide change which disrupts a *Hha* I restriction enzyme cleavage site (GCGC to GCAC) at nucleotide 260 of the 462 bp PCR fragment produced by primers G9-1734 and G9-2175 above. Digestion was carried out using 9 µl of PCR product and 3 U *Hha* I in 15 µl final volume, for 6 h at 37° C. Restriction digestion of the PCR product from wildtype animals with *Hha* I resulted in cleavage of the 462 bp product (at two internal *Hha* I sites) into fragments of 52 bp, 156 bp and 254 bp. However, DNA fragments containing the A nucleotide are not cleaved at this site and fragment sizes of 52 bp and 410 bp are seen. Animals heterozygous for the mutation have fragments of all four sizes (52 bp, 156 bp, 254 bp and 410 bp).

The remaining single nucleotide polymorphisms (SNPs) in GDF-9 and GDF-9B identified by sequencing did not affect common restriction endonuclease cleavage sites. In order to screen these polymorphisms through the F700 Belclare and Cambridge flocks of sheep, PCR was carried out using primers with single mismatches in order to deliberately generate products that contained restriction enzyme sites. Assays were designed so that digestion with the appropriate restriction enzyme cleaved either PCR products from wild-type animals or PCR products from animals containing the SNP, as specified below. The resulting band shift was resolved on a high percentage agarose gel.

The primer sequences and PCR conditions for each assay were as follows. The mismatch created in the appropriate primer to generate the restriction enzyme cleavage site is underlined.

In all five assays below, amplification was carried out at: 94°C for 5 min; 35 cycles of 94°C for 30 sec, an annealing step for 40 sec (at the specific temperature stated below for each assay) and 72°C for 30 sec; followed by a final extension of 72°C for 4 min. Magnesium concentration was 1.5 mM.

1. The primers used for the GDF-9 [324] nucleotide change amplify a 161 bp PCR product were:

[324]-Sfu1F 5'-GGAATATTCACATGTCTGTAAATTTTACATGTTCG-3'

[324]-Sfu3R 5'-GAGGGAATGCCACCTGTGAAAAGCC-3'

The annealing temperature was 63°C.

The non-wildtype strand was cleaved by restriction enzyme *Sfu* I.

2. The primers used for the GDF-9 [714] nucleotide change amplify a 158 bp PCR product were:

[714]-Tru1R 5'-CAGTATCGAGGGTTGTATTTGTGTGGGGCCT-3'

[714]-Tru3F 5'-GCCTCTGGTTCCAGCTTCAGTC-3'

The annealing temperature was 63°C.

The non-wildtype strand was cleaved by restriction enzyme *Mse* I.

3. The primers used for the GDF-9 [787] nucleotide change amplify a 139 bp PCR product were:

[787]-Dde1R: 5'-CATGGATGATGTTCTGCACCATGGTGTGAACCTGA-3'

[787]-Dde3F: 5'-CTTTAGTCAGCTGAAGTGGGACAAC-3'

The annealing temperature was 62°C.

The wildtype strand was cleaved by restriction enzyme *Dde* I.

4. The primers used for the GDF-9B [S1] nucleotide change amplify a 141 bp PCR product were:

[S1]-Hinf1F: 5'-CACTGTCTTCTTGTTACTGTATTTCAATGAGAC-3'

B26: 5'-GATGCAATACTGCCTGCTTG-3'

The annealing temperature was 63°C.

The wildtype strand was cleaved by restriction enzyme *Hinf* I.

5. The primers used for the GDF-9B [S2] nucleotide change amplify a 153 bp PCR product were:

[S2]-Dde1F: 5'-GCCTTCCTGTGTCCCTTATAAGTATGTTCCCCTTA-3'

O4: 5'-TTCTTGGGAAACCTGAGCTAGC-3'

The annealing temperature was 64°C.

The wildtype strand was cleaved by restriction enzyme *Dde* I.

Restriction digestion of PCR with *Hinf* I [S1] or *Dde* I [[787] and [S2]] resulted in a cleavage of the longer primer from the fragment amplified from wild-type alleles (thus producing a 30-35 bp smaller product than the uncleaved fragment from animals containing alleles with the mutation). Restriction digestion of PCR with *Sfu* I [324] or

Mse I [714] resulted in a cleavage of the longer primer from the fragment amplified from mutant alleles (thus producing a 30-35 bp smaller product than the uncleaved fragment from animals containing wild-type alleles). Animals heterozygous for any of the mutations had fragments of both sizes. The digested fragments were separated on a 4% agarose gel and visualised with ethidium bromide staining. The gels were scored for the presence or absence of the mutations. Homozygous, heterozygous and negative controls were included with each assay.

Immunisation experiments

All experiments were performed with the approval of the Animals Ethics Committee at Wallaceville Animal Research Centre in accordance with the 1987 Animal Protection (Codes of Ethical Conduct) Regulations of New Zealand. The animals used in the immunization studies (n=54) were 5 to 6 year old parous Romney ewes.

Generation of antigens for immunization of sheep

Peptides KKPLVPASVNLSEYFC (GDF-9) and SEVPGPSREHDGPESC (GDF-9B) were synthesized and conjugated to KLH through the C terminal cysteine residue by Macromolecular Resources (Colorado State University, Fort Collins, CO).

Long-term active immunization of ewes against GDF-9 and GDF-9B peptides

Ewes were injected (i.m) with 0.4 mg KLH (control, n=10), 0.4 mg KLH-GDF-9 peptide conjugate (GDF-9 peptide; n=10) or 0.4 mg KLH-GDF-9B peptide conjugate (GDF-9B peptide; n=10) in 1 ml of Freund's complete adjuvant for the initial immunization. Thereafter, ewes were immunized once monthly with 0.2 mg KLH, GDF-9 peptide or GDF-9B peptide in 1 ml of saline mixed with 1.25 ml STM (Span-Tween-Marcol) for 6 months. After the 5th injection, vasectomised rams with marking harnesses were run with the ewes to monitor estrous cycles. The length of the estrous

cycle was calculated as the days between first observed markings by the vasectomised ram of successive cycles. In addition, blood samples were collected via the jugular vein 3 times a week for determination of plasma progesterone concentrations. Ovulation rates of the ewes that displayed estrous behaviour were determined by laparoscopy once all of the control ewes had been observed in estrus and for each successive estrous cycle. In addition, ovulation rate of all ewes was determined by laparoscopy 3-4 weeks prior to ovarian collection. Approximately 2 weeks following the final injection, ewes were killed using a captive bolt and exsanguinated. The blood collected from all ewes was to be used in subsequent passive immunisation studies. Both ovaries were recovered and the number of corpora lutea present was recorded and one ovary from each ewe was fixed in Bouins fluid for morphological examination and analysis of follicular populations.

Passive immunisation of ewes against KLH, KLH-GDF-9 peptide and KLH-GDF-9B peptide

Pools of antiplasma from KLH (n=9), GDF-9 peptide (n=7, all anovulatory ewes) and GDF-9B peptide (n=9, all anovulatory ewes) treated ewes were generated by combining the plasmas obtained from some of the actively immunized ewes within each treatment group. The estrous cycles of ewes were synchronized by using a prostaglandin $F_{2\alpha}$ derivative (Estrumate; 125μg). Estrus was detected with the aid of a vasectomised ram wearing a marking harness. On day 4 or 5 of the estrus cycle (estrus = day 0) ewes were laparascoped to determine ovulation rate and fitted with an indwelling jugular cannula. The following day ewes (n=4-5 per group) were administered 100 ml of antiplasma to KLH, GDF-9 peptide or GDF-9B peptide through the indwelling jugular cannula. Ewes were given another injection of Estrumate, at 96h after administration of the antiplasma to induce a follicular phase and ovulation rate was determine by laparoscopy at 10 days after the injection of Estrumate and every 15-18 days thereafter until the end of the

breeding season (as assessed by lack of estrous activity in non-experimental sheep). Blood samples were collected from the ewes at 5 minutes, 1 h and 96 h after injection of the antiplasma and thereafter 3 times a week from the 2nd injection of Estrumate for determination of antibody titers and concentrations of progesterone in plasma.

Determination of progesterone concentrations

Concentrations of progesterone in plasma were determined by radioimmunoassay (RIA). The inter- and intra-assay co-efficients of variation were <10% and assay sensitivity was 0.1 ng/ml. All samples below the sensitivity of the assay were assigned a value of 0.1 ng/ml for statistical analysis.

Short-term immunisations

Romney ewes were immunized with KLH (N=50), KLH conjugated to GDF-9 peptide (N=30) or KLH conjugated to GDF-9B peptide (N=30). The antigens were administered in DEAE Dextran (4% w/v) on 2 occasions one month apart. The number of corpora lutea (CL) was determined following the first observed oestrus which occurred after the booster immunization. In addition, in a selected subpopulation of these ewes (N=26 KLH, N=15 GDF-9, N=16 GDF-9B) the number of CL present following the next oestrus was also determined. The average number of CL for each ewe was analysed by Chi-square analysis.

Statistical analysis

For the long-term, actively immunized ewes, ovulation rate for individual ewes was calculated as the mean of the number of corpora lutea observed at all observations for that ewe when at least 1 corpus luteum (CL) was present (i.e. observations of no CL were excluded from the calculation). The Kruskal-Wallis test was used to compare ovulation rates between the KLH-GDF-9B mature protein and the KLH treated groups.

No other groups were included in this comparison since none had sufficient numbers of ewes ovulating. The Chi Square test was used to compare the proportion of ewes observed in estrus by the time all the control ewes had been observed in estrus. In addition the Chi Square test was used to compare the proportion of ewes with corpora lutea on their ovaries 3-4 weeks before and at ovarian collection.

When examining the effects of active immunization treatments on ovarian volumes, numbers of follicles or oocyte or follicular diameters, the data were analysed within each follicle type after normalising the data by log transformation. For each parameter a one-way ANOVA was performed, after blocking on animals where appropriate, and differences between treatment groups were determined by least significant difference.

For the passively immunized ewes, differences in the number of ewes with corpora lutea at each laparoscopy were determined using Fisher's exact test. The areas under the curves were calculated using Genstat using the area function for progesterone values from 2 to 19 days following injection of Estrumate that was given 4 days after administration of plasma. Resulting values were analysed with one-way ANOVA and differences between the control and treated ewes determined with Fisher's pairwise comparisons.

For the short term active immunizations, ovulation rate for individual ewes was calculated as the average of the number of corpora lutea observed at both observations. Data was analysed using the general linear models procedures of SAS. Differences between least-squares means were evaluated by least significant differences.

RESULTS and ANALYSIS OF RESULTS

Finding mutations in Cambridge and F700 Belclare animals

In order to determine whether mutations in GDF-9 or GDF-9B were contributing to sterility in these animals sequence information was obtained for the entire coding sequence of both genes in a subset of Irish Cambridge (N = 9) and F700 Belclare sheep (N = 10). Animals were chosen for full-length sequencing based on their sterility phenotype or their pedigree relationship to sterile animals. In addition, mutation detection was also carried out by single-stranded conformational polymorphism (SSCP) analysis independently of the above sequencing in F700 Belclare pedigrees (23 animals and 58 progeny test daughters of three rams) and also on 2 Cambridge rams.

Mutations in GDF-9

Sequence of GDF-9 revealed eight single nucleotide polymorphisms across the entire coding region (Table 1, Figure 4). SSCP analysis identified five fragments across the gene which contained conformational differences. These differences correspond to one single nucleotide polymorphism (SNP) in exon 1, one SNP in the intron and five SNPs in exon 2.

Original naming of the mutations (numbers in square brackets [ ], Table 1) refers to the nucleotide position from the start of exon 2, except for [E1] which refers to the polymorphism found in exon 1 of GDF-9. Table 1 shows the relationship between (a) the original numbering system, (b) the coding nucleotide position in the full length coding sequence numbered from the first atg, (c) the position of the coding amino acid residue involved, and (d) the position of the coding residue (if any) within the mature coding sequence, numbering from the first amino acid after the furin protease processing site. For example mutation [581] is a G to A nucleotide substitution at

coding nucleotide 978 of GDF-9 which corresponds to an unchanged glutamate (Glu) coding residue 326 of the full length unprocessed protein, or residue 8 of the processed mature peptide.

Three of the eight polymorphisms are nucleotide changes which do not result in an altered amino acid ([74] at nucleotide position 471, [80] at nucleotide 477, and [581] at nucleotide position 978). The five remaining nucleotide changes [324], [597], [714], and [787] gave rise to amino acid changes (Table 1), Figure 1, Figure 4), although three of them were relatively conservative changes. The [E1] arginine to histidine change at amino acid residue 87 in exon 1 substituted one basic charged polar group with another, and occurred at a position prior to the furin processing site, so was unlikely to affect the activity of the mature protein. Both the [597] valine to isoleucine change at amino acid residue 332 of the unprocessed protein (residue 14 of the mature coding region) and the [714] valine to methionine at residue 371 of the unprocessed protein (residue 53 of the mature coding region) substituted non-polar groups with non-polar groups. The remaining two changes resulted in non-conservative substitutions. The [324] glutamic acid to lysine change at amino acid residue 241 of the unprocessed protein replaced an acidic group with a basic group, but this occurred at a position prior to the furin processing site and was unlikely to affect the mature active coding region. However the [787] serine to phenylalanine change at residue 395 replaced an uncharged polar group with a non-polar group at residue 77 of the mature coding region. The nucleotide and amino acid changes are illustrated in Figure 1 and Figure 4.

Mutations in GDF-9B

Both DNA sequencing and independent SSCP analysis of GDF-9B in Cambridge and F700 Belclare sheep revealed four polymorphisms across the entire coding region (Table 1, Figure 5). Original naming of these mutations (in square brackets [ ]) refers

specifically to the leucine deletion [Leu], or for the conservative [422] T to C mutation, the nucleotide position from the start of exon 2. GDF-9B mutations which changed amino acids were named [S1] and [S2]. Table 1 shows the relationship between (a) the original numbering system, (b) the coding nucleotide position in the full length coding sequence numbered from the first atg, (c) the position of the coding amino acid residue involved, and (d) the position of the coding residue (if any) within the mature coding sequence, numbering from the first amino acid after the furin protease processing site. For example mutation [S2] was a G to T nucleotide substitution at coding nucleotide 1100 of GDF-9B which corresponds to an serine (Ser) residue changing to an isoleucine (Ile) residue at coding residue 367 of the full length unprocessed protein, or residue 99 of the processed mature peptide.

The first of these four polymorphisms (Table 1) was a previously-reported leucine deletion polymorphism [leu] in the predicted signal sequence (Galloway *et al.*, 2000) whereby some sheep have two leucine codons (CTT) at this position and some sheep have only one. This polymorphism has been shown to be unrelated to fertility and ovulation rate in Inverdale sheep (Galloway *et al.*, 2000). One other nucleotide change, [422], did not result in an altered amino acid (nucleotide position 747). The remaining two nucleotide changes ([S1] and [S2]) gave rise to more critical changes in the protein (Figure 2, Figure 5). The [S1] C to T change at nucleotide 718 introduced a premature stop codon (TAG) in the place of glutamic acid (Q, CAG) at amino acid residue 239 of the unprocessed protein, which presumably resulted in complete loss of GDF-9B function. The [S2] G to T change at nucleotide 1100 changed the serine residue at amino acid 99 of the mature active protein (residue 367 of the unprocessed protein) to an isoleucine, thereby substituting an uncharged polar group with a nonpolar group. The nucleotide and amino acid changes are illustrated in Figure 2 and Figure 5.

Screening for mutations in more animals

Initial sequencing of a smaller number of animals from each family identified the [74], [80], [324], [714], and [787] nucleotide changes in GDF-9, and the [S1], [S2] and [422] changes in GDF-9B. Forced RFLP (restriction fragment length polymorphism) assays to detect the specific SNPs were developed for [324], [714], [787] (GDF-9) and for [S1] and [S2] (GDF-9B), and these assays were carried out on larger numbers of animals (Table 2). Subsequent sequencing of full length GDF-9 and GDF-9B in more animals revealed the [581] and [597] nucleotide changes in GDF-9 in the Cambridge sheep but not the F700 Belclares. Independent SSCP analysis identified the [E1] polymorphism in exon 1 of GDF-9 in one ram, and this was also screened through further animals. [E1] was found to be associated with the wildtype alleles in this ram and his backcross progeny, and not associated with ovulation rate.

Homozygous mutations relate to sterility

Presence or absence of each of these nucleotide changes was analysed in relation to sterility or fertility in all of the animals tested, revealing that only the [787] change in GDF-9 and the [S1] and [S2] changes in GDF-9B contributed to infertility. Female sheep which were homozygous for [787] were sterile; female sheep which were homozygous for [S1], or homozygous for [S2] were sterile; female sheep which were heterozygous for [S1] and [S2] simultaneously (whereby both copies of the X chromosome carried a different GDF-9B mutation) were sterile. Figures 3a and 3b show data from small pedigrees illustrating what was seen in the larger set of animals.

Figure 3a illustrates a F700 Belclare pedigree. The sire R830 carried the GDF-9B [S2] mutation on his X chromosome and a single copy of the GDF-9 [787] mutation on chromosome 5, but did not have the GDF-9B [S1] mutation. Dam 9704 carried a single copy of the GDF-9B [S1] mutation on her X chromosome and their two female

offspring (930458 and 930459) were sterile since they had inherited mutated copies of GDF-9B from both parents. Dam 8783 carried a single copy of the GDF-9 [787] mutation on chromosome 5 and the female offspring of her mating with sire R830 were infertile and were homozygous for the GDF-9 [787] mutation. Their infertility cannot be explained by GDF-9B mutations. Offspring 930810 and 948302 were not homozygous for any of these mutations and hence were fertile. All three functional mutations ([S1], [S2], and [787]) were seen in the F700 Belclare flock (Table 2).

Figure 3b illustrates two Cambridge pedigrees. The sire 962101 carried the GDF-9B [S1] mutation on his X chromosome and a single copy of the GDF-9 [787] mutation on chromosome 5, but did not have the GDF-9B [S2] mutation. Dam 962152 carried a single copy of the [S1] mutation on her X chromosome and a single copy of the [787] mutation on chromosome 5. Their two female offspring (997634 and 997635) were sterile and had inherited mutated copies of both GDF-9B ([S1]) and GDF-9 ([787]) from both parents. Dam 976234 only carried a single copy of the [S1] mutation and one female offspring (997553) was infertile, having inherited mutated copies of GDF-9B ([S1]) from both parents, whereas 997552 was fertile. Sire 930142 was homozygous for the GDF-9 [787] mutation and carried the GDF-9B [S1] mutation on his X chromosome, whereas dam 8874 was only heterozygous for the GDF-9 [787] mutation and carried no GDF-9B mutation. Their daughter (948093) had inherited two copies of the GDF-9 [787] mutation and was sterile even though she was also heterozygous for the GDF-9B [S1] mutation which she inherited from her sire. The [S2] mutation was not seen in any animals tested from the Cambridge flock (Table 2).

Among the animals tested for these changes, fertile animals homozygous for both GDF-9 [324] and [714] were found and it is therefore concluded that neither of those changes result in disruption of the genes sufficient to cause sterility. Animals were also found

which were heterozygous for GDF-9 and GDF-9B mutations together, and these animals were not sterile.

Structural effects of mutations on activity

Structural data is available for members of the TGFß superfamily which may provide information about the likely effects of each of the three mutations ([S1], [S2] and [787]) on the biological activity of GDF-9 and GDF-9B, and hence explain the association with sterility. Structures have been reported for TGF-ß1 (Hinck *et al.*, 1996); TGF- ß2 (Daopin *et al.*, 1992), TGF-ß3 (Mittl *et al.*, 1996), BMP7/OP1 (Griffith *et al.*, 1996) and BMP2 (Scheufler *et al.*, 1999). Receptor binding structures have also been reported for BMP2 with the BRIA receptor binding ectodomain (Kirsch *et al.*, 2000) and for TGF-ß3 with the TßR2 receptor binding ectodomain (Hart *et al.*, 2002).

In the present invention, the [S1] mutation resulted in premature termination of GDF-9B protein prior to the mature active protein processing site. It is thus expected that this mutation would result in no mature protein being translated, and appears to be an even more severe effect than the Hanna mutation (Galloway et al., 2000) which results in infertility in sheep. The GDF-9B [S2] mutation changed an uncharged polar serine residue (residue 99 of mature GDF-9B) which is conserved across most members of the TGFß superfamily, to a non-polar isoleucine (Figure 6). This serine (and the nearby conserved leucine) has been shown to be essential for receptor binding by structural and site-directed mutagenesis studies of BMP2. In F700 Belclare sheep it appears that this mutation abolishes biological activity of GDF-9B, possibly by affecting receptor binding. The GDF-9 [787] mutation changed an uncharged polar serine residue (residue 77 of mature GDF-9) to a non-polar phenylalanine in a region of the molecule which appears to be involved in dimerisation. This change occurred only three residues away from a conserved histidine (H80) of the mature GDF-9 peptide (Figure 6). In BMP7 this

conserved histidine exhibits hydrogen bonding to three residues of the paired molecule in the BMP7 dimer (Griffith *et al.*, 1996) and TGFß3 (Mittl *et al.*, 1996). GDF-9 lacks the interchain disulphide bond which forms a covalent link between both monomers of the biologically active dimer that is found in most other members of the TGFß superfamily. It is therefore likely that in GDF-9 the hydrogen bonds between monomers would be even more critical for maintaining dimer stability, so that the GDF-9 [787] mutation possibly abolished biological activity by disrupting dimerisation.

Heterozygous animals have increased ovulation rate

Irish Cambridge and F700 Belclare sheep have increased ovulation rates as well as infertility (Hanrahan, 1996). Ovulation rate data of fertile ewes which had been genotyped for the [S1], [S2] and [787] mutations was collected (Table 3). Heterozygous carriers of mutations in GDF-9B (either [S1] or [S2]) showed an increased ovulation rate similar to those seen in Inverdale and Hanna sheep (Davis *et al.*, 2001). Animals which were heterozygous for both a GDF-9B mutation and a GDF-9 mutation had an even higher ovulation rate, and this effect appeared to be additive.

The additive effects of the GDF-9 mutation and GDF-9B mutations together in one animal imply that GDF-9 and GDF-9B work independently so that a combination of both proteins would alter ovarian function more effectively than by altering either GDF-9 or GDF-9B alone.

Effect of immunising sheep against GDF-9

In addition to the genotype effects above, both long-term active immunisation and short-term passive immunisation of sheep with GDF-9 was carried out and shown to cause sterility and/or abnormal corpus luteum function. This finding provides additional evidence that a homozygous GDF-9 mutant phenotype is one of sterility.

Ewes were actively immunised against KLH (control, n = 9) or KLH conjugated to GDF-9 peptide (n = 10). The ewes actively immunized with the GDF-9 peptide showed no cyclic estrous behaviour (Table 4) as evidenced by repeated laproscopy. High (normal) progesterone concentrations were only seen in one or two samples (Figure 7). Generally, when corpora lutea and/or luteal-like structures were observed following long-term immunisation against KLH conjugated to GDF-9 peptide, progesterone concentrations were abnormal. In addition many of the ewes did not have any visible antral follicles at laparoscopy or at ovarian collection. Figure 7 also shows data for ewes immunised against GDF-9B/(BMP15) for comparison.

Passive immunisation using 100 ml of GDF-9 peptide antiplasma caused abnormal luteal function within 30 days of administration of the antiplasma (Figure 8). There were no differences in ovulation rates among the groups before administration of the antiplasma. Administration of antiplasma against GDF-9 peptide 4 days before induction of the follicular phase did not affect ovulation rate. However, at laparoscopy the corpora lutea of two of the animals treated with GDF-9 antiplasma appeared smaller than normal. In addition, the overall mean concentration of progesterone during the subsequent luteal phase was less ($P<0.05$) than that observed in the control animals (Figure 8). This was the result of the progesterone concentrations being normal in two of the animals but in the other three animals, the post ovulation rise in progesterone was delayed even though luteolysis occurred at the normal time. Figure 8 also shows data for passive immunisation with GDF-9B/(BMP15) for comparison.

The finding of abnormal luteal function following GDF-9 immunisation (both passive and active) has not been previously reported. It is postulated, therefore, that the administration of supplementary GDF-9 and GDF-9B, or analogue thereof, or GDF-9 or GDF-9B antagonists, may modify corpus luteum function.

In a further experiment, short-term active immunisation of sheep with GDF-9 or GDF-9B was shown to mimic the heterozygous effects of mutations in these genes. Short-term immunisation using milder adjuvant than in the previous experiments (2 immunisations in DEAE-Dextran adjuvant), with either KLH conjugated to GDF-9 peptide or with KLH conjugated to GDF-9B peptide, acted to increase ovulation rate in the animals which ovulated as measured by the number of corpora lutea (CL) (Table 5).

The observation that an increased ovulation rate effect (ie as seen in heterozygous carriers of these inactivating mutations) can also be induced in sheep by short-term active immunisation against peptides of GDF-9 and GDF-9B provides new methods for altering ovarian function.

Temporary/reversible effects in immunization with GDF-9B

Four of the 30 ewes immunised (0.4 mg GDF-9B peptide-KLH followed by 0.2 mg GDF-9B peptide-KLH 30 days apart) displayed transient infertility. After the second immunization, ewes failed to show oestrus and upon examination of their ovaries, failed to show any evidence of ovulation (no corpora lutea). These ewes returned to estrous over the next 30 days and were placed with a fertile ram. The oocytes release at oestrous were healthy as indicated by the following. Three of the four ewes were flushed to recover embryos for transfer as they had higher ovulation rates upon return to fertility. While no embryos were recovered from one of the ewes, developing embryos were recovered from the other two ewes. In addition, while the embryos from one of the ewes were too advanced for recovery (by approximately one week) the two embryos from the other ewe were transferred to a recipient ewe and resulted in the birth of one lamb. The fourth ewe was allowed to carry the pregnancy to term and gave birth to two lambs, one stillborn and one live. These results provide evidence that the sterility induced by the

method of the present invention can be temporary or reversible and may therefore be used in methods of contraception.

CONCLUSIONS

These findings provide the first evidence that mutations in GDF-9 and GDF-9B are associated with the reproductive effects seen in the Cambridge and Belclare breeds of sheep. The increased ovulation rate and sterility phenotypes in these animals can be explained by the presence of heterozygous mutations and homozygous mutations, respectively, in these genes. Methods which are able to induce changes in the biological activity of GDF-9 and/or GDF-9B to mimic these genotypes have been developed to modulate fertility in mammals in need thereof.

INDUSTRIAL APPLICATION

The present invention provides compositions and methods for modulating the ovulation rate and therefore fertility in female mammals including humans.

TABLE 1

Sequence variations in GDF-9 and GDF-9B within the Irish Cambridge and F700 Belclare F700 flocks

Gene	[original name]	nucl. change	coding nucl.(bp)	coding residue	mature residue	result
GDF-9	[E1]	G-A	260	87		Arg (R) – His (H)
	[74]	C-T	471	157		unchanged Val (V)
	[80]	G-A	477	159		unchanged Leu (L)
	[324]	G-A	721	241		Glu (E) – Lys (K)
	[581]	A-G	978	326	8	unchanged Glu (E)
	[597]	G-A	994	332	14	Val (V) – Ile (I)
	[714]	G-A	1111	371	53	Val (V) – Met (M)
	[787]	**C-T**	**1184**	**395**	**77**	**Ser (S) – Phe (F)**
GDF-9B	[Leu]	CTT del	28-30	10		Leu deletion
	[S1]	**C-T**	**718**	**239**		**Gln (Q) – STOP**
	[422]	T-C	747	249		unchanged Pro (P)
	[S2]	**G-T**	**1100**	**367**	**99**	**Ser (S) – Ile (I)**

Columns indicate the relationship between (a) the original naming system used for each polymorphism, (b) the nucleotide change, (c) the coding nucleotide position (in base

pairs (bp)) in the full length coding sequence numbered from the first atg start codon, (d) the position of the coding amino acid residue involved (starting from the first Met residue), and (e) the position of the coding residue (if any) within the mature coding sequence, numbering from the first amino acid after the furin protease processing site. For example mutation [581] is a G to A nucleotide substitution at coding nucleotide 978 of GDF-9 which corresponds to an unchanged glutamate (Glu) coding residue 326 of the full length unprocessed protein, or residue 8 of the processed mature peptide. Polymorphisms associated with infertility and ovulation rate traits are in bold.

TABLE 2. Genotype analysis of nucleotide changes in GDF-9B and GDF-9 genes from Irish Cambridge and F700 Belclare sheep.

Numbers shown are the number of individuals carrying at least one copy of the given mutation with the total number of individuals genotyped indicated in brackets underneath. Genotypes were determined by specific SNP assay and/or sequencing.

* The [E1] polymorphism in exon 1 of GDF-9 (see Table 1) was identified by SSCP analysis and was not tested in the same set of animals used for the above table.

	GDF-9B			GDF-9*						
	[S1]	[S2]	[422]	[74]	[80]	[324]	[581]	[597]	[714]	[787]
F700 Belclares	9 (83)	71 (86)	2 (13)	6 (10)	6 (10)	13 (29)	0 (10)	0 (10)	2 (19)	11 (86)
Cambridge	74 (129)	0 (131)	0 (9)	0 (9)	7 (9)	1 (26)	3 (9)	2 (9)	7 (24)	95 (126)

TABLE 3. Least squares means for ovulation rate of sheep carrying the different genotypes for GDF-9 and GDF-9B mutations

Genotype			*Breed*	
GDF-9B		**GDF-9**	**F700 Belclare**	**Cambridge**
[S1]	**[S2]**	**[787]**		
0	0	0	1.92±0.277 (n = 11)	2.27±0.488 (n = 10)
0	0	1	2.67±0.895 (n = 1)	4.39±0.308 (n = 28)
0	1	0	3.26±0.184 (n = 32)	-
0	1	1	6.09±0.549 (n = 3)	-
1	0	0	2.69±0.475 (n = 4)	3.11±0.438 (n = 15)
1	0	1	-	5.77±0.270(n = 38)
Effect of GDF-9B [S1]			0.77±0.537 (P = 0.16)	1.18±0.387 (P<0.01)
Effect of GDF-9B [S2]			2.38±0.548 (P<0.01)	-
Effect of GDF-9 [787]			1.79±0.548 (P<0.01)	2.35±0.392 (P<0.01)

(n) = no. of ewes

TABLE 4. Proportions of ewes immunized against KLH, GDF-9 peptide or GDF-9B peptide in estrus at the time of first laparoscopy (1^{st}), with visible luteal structures at laparoscopy 3-4 weeks before collection (2^{nd}) and at ovarian collection (3rd).

Immunized Group	1^{st}	2^{nd}	3^{rd}
KLH	9/9	9/9	9/9
GDF-9 peptide	2/10*	2/10*	3/10*
GDF-9B peptide	1/10*	1/10*	1/10*

*Signifies a value that is different from the control (KLH) value (P<0.05)

TABLE 5. Short-term immunisation of sheep with GDF-9 or GDF-9B

Number of CL	**1 to < 2**	**2 to < 3**	**=3**
Treatment	**Number of CL recorded in treated ewes**		
KLH (n=50)	**21**	**29**	**0**
GDF-9 peptide* (n=30)	**7**	**17**	**6**
GDF-9B peptide* (n=30)	**2**	**18**	**10**

* Using Chi-square analysis, the GDF-9 and GDF-9B immunized ewes have significantly higher ovulation rates (as measured by corpora lutea (CL) than KLH control ewes ($P<0.01$).

Aspects of the present invention have been described by way of example only and it should be appreciated that modifications and additions may be made thereto without departing from the appended claims.

REFERENCES

Aaltonen J., Laitinen M., Vuojolainen K., Jaatinen R., Horelli-Kuitunen N., Seppa L., Louhio H., Tuuri T., Sjoberg J., Butzow R., Hovatta O., Dale L., Ritvos O (1999) Human growth differentiation factor- 9 (GDF-9) and its novel homolog GDF-9B are expressed during early folliculogenesis. J Clin Endocrinol Metab 84: 2744-2750.

Bodensteiner KJ., Clay C.M., Moeller C.L., and Sawyer HR., (1999) Molecular cloning of the ovine growth/differentiation factor-9 gene and expression of growth/differentiation factor-9 in ovine and bovine ovaries. Biology of Reproduction 60, 381-386.

Bodensteiner, K.J., McNatty, K.P., Clay, C.M., Moeller, C.L. and Sawyer, H.R. (2000) Expression of growth and differentiation factor-9 in the ovaries of fetal sheep homozygous or heterozygous for the Inverdale prolificacy gene (*FecX*^{I}). Biology of Reproduction 62: 1479-1485.

Daopin S., Piez KA., Ogawa Y., Davies DR (1992) Crystal structure of transforming growth factor-beta 2: an unusual fold for the superfamily. Science 257:369-373.

Davis GH., Bruce GD., & Dodds KG (2001) Ovulation rate and litter size of prolific Inverdale (FecX^{I}) and Hanna (FecX^{H}) sheep. Proceedings of Association for the Advancement of Animal Breeding and Genetics 14: 175-178.

Dong J., Albertini DF., Nishimori K., Kumar TR., Lu N., Matzuk MM (1996) Growth differentiation factor-9 is required during early ovarian folliculogenesis. Nature 383:531-535.

Dube JL., Wang P., Elvin J., Lyons KM., Celeste AJ., Matzuk MM (1998) The bone morphogenetic protein 15 gene is X-linked and expressed in oocytes. Mol Endocrinol 12:1809-1817.

Eckery DC., Whale LJ., Lawrence SB., Wilde KA., McNatty K.P., & Juengel., J.L. (2002). Expression of mRNA encoding growth differentiation factor 9 and bone morphogenetic protein 15 during follicular formation and growth in a marsupial, the brushtail possum (*Trichasurus vulpecula*). Molecular & Cellular Endocrinology, 192: 115-126.

Galloway SM., McNatty KP., Cambridge LM., Laitinen MPE., Juengel JL., Jokiranta TS., McLaren RJ., Luiro K., Dodds KG., Montgomery GW., Beattie AE., Davis GH., Ritvos O (2000) Mutations in an oocyte-derived growth factor gene (*BMP15*) cause increased ovulation rate and infertility in a dosage-sensitive manner. Nature Genetics 25:279-283.

Griffith DL., Keck PC., Sampath TK., Rueger DC., Carlson WD (1996) Three-dimensional structure of recombinant human osteogenic protein 1: structural paradigm for the transforming growth factor beta superfamily. Proc Natl Acad Sci U S A 93:878-883.

Hart PJ., Deep S., Taylor A.B., Shu Z., Hinck C.S., and Hinck A.P (2002). Crystal structure of the human TßR2 ectodomain-TGF-ß3 complex. Nature Structural Biology 9: 203-208.

Hanrahan JP (1991) Evidence for single gene effects on ovulation rate in the Cambridge and Belclare breeds. In *Major Genes for Reproduction in Sheep* pp 93-102 Eds J.M. Elsen, L.Bodin and J. Thimonier, INRA, Paris.

Hinck AP., Archer SJ., Qian SW., Roberts AB., Sporn MB., Weatherbee JA., Tsang ML., Lucas R., Zhang B-L., Wenker J., and Torchia DA (1996) Transforming growth factor ß1: Three-dimensional structure in solution and comparison with the X-ray structure of transforming growth factor ß2. Biochemistry 35: 8517-8534.

Jaatinen R., Laitinen MP., Vuojolainen K., Aaltonen J., Louhio H., Heikinheimo K., Lehtonen E., and Ritvos O (1999) Localisation of growth differentiation factor-9 (Gdf-9) mRNA and protein in rat ovaries and cDNA cloning of rat GDF-9 and its novel homolog GDF-9B.Mol Cell Endocrinol 156: 189-193.

Kirsch, T., Sebald, W. and Dreyer, M.K. (2000a). Crystal structure of the BMP2-BRIA ectodomain complex. Nature Structural Biology 7: 492-496.

Laitinen M., Vuojolainen K., Jaatinen R., Ketola I., Aaltonen J., Lehtonen E., Heikinheimo M., Ritvos O (1998) A novel growth differentiation factor-9 (GDF-9) related factor is co-expressed with GDF-9 in mouse oocytes during folliculogenesis. Mech Dev 78:135-140.

McGrath SA., Esquela AF., Lee SJ (1995): Oocyte-specific expression of growth differentiation factor-9. Mol Endocrinol 9:131-136.

McPherron AC., Lee SJ: GDF-3 and GDF-9 (1993) two new members of the transforming growth factor-beta superfamily containing a novel pattern of cysteines. J Biol Chem 268:3444-3449.

Mittl PRE., Priestle JP., Cox DA., McMaster G., Cerletti N., and Grutter MG., (1996) The crystal structure of TGF-ß3 and comparison to TGF-ß3: Implications for receptor binding. Protein Science 5: 1261-1271.

Sadighi M., Bodensteiner KJ., Beattie AE. and Galloway SM (2002). Genetic mapping of ovine growth differentiation factor 9 (*GDF-9*) to sheep chromosome 5. Animal Genetics, 33: 244-245.

Scheufler C., Sebald W., Hulsmeyer M (1999) Crystal structure of human bone morphogenetic protein-2 at 2.7 A resolution. J Mol Biol 287:103-115.

Yan C., Wang P., DeMayo J., DeMayo FJ., Elvin J., Carino C., Prasad SV., Skinner SS., Dunbar BS., Dube JL., Celeste AJ. and Matzuk MM (2001) Synergistic roles of bone morphogenetic protein 15 and growth differentiation factor 9 in ovarian function. Molecular Endocrinology 15: 854-866.